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The opine synthase genes carried by Ti plasmids contain all signals necessary for expression in plants

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Signals necessary for *in vivo* expression of Ti plasmid T-DNA-encoded octopine and nopaline synthase genes were studied in crown gall tumors by constructing mutated genes carrying various lengths of sequences upstream of the 5′ initiation site of their mRNAs. Deletions upstream of position −294 did not interfere with expression of the octopine synthase gene while those extending upstream of position −170 greatly reduced the gene expression. The estimated size of the octopine synthase promoter is therefore 295 bp. The maximal length of 5′ upstream sequences involved in the *in vivo* expression of the nopaline synthase gene is 261 bp. Our results also demonstrated that Ti plasmid-derived sequences contain all signals essential for expression of opine synthase genes in plants. Expression of these genes, therefore, is independent of the direct vicinity of the plant DNA sequences and is not activated by formation of plant DNA and T-DNA border junction.

Key words: Agrobacterium tumefaciens/Ti plasmids/opine synthase genes/promoter regions

Introduction

Crown gall, a neoplastic disease of dicotyledonous plants, develops after infection of wounded tissue with *Agrobacterium tumefaciens* strains carrying large tumor-inducing (Ti) plasmids (Zaenen *et al.*, 1974; Van Larebeke *et al.*, 1974; Watson *et al.*, 1975). A well-defined segment (T-region) of the Ti plasmid is transferred and covalently integrated, without rearrangements, in plant nuclear DNA (Chilton *et al.*, 1977, 1980; Schell *et al.*, 1979; Thomashow *et al.*, 1980; Lemmers *et al.*, 1980; Zambryski *et al.*, 1980; Yadav *et al.*, 1980; Willmitzer *et al.*, 1980). The transferred DNA (T-DNA) is transcribed (Drummond *et al.*, 1977; Willmitzer *et al.*, 1981a; Gelvin *et al.*, 1981) by the host RNA polymerase II (Willmitzer *et al.*, 1981b).

Transformed crown gall cells are capable of autonomous growth in the absence of exogenous phytohormones (Braun, 1956). Moreover, these plant tumors synthesize a variety of low mol. wt. metabolites (termed opines) which are characteristic for Ti plasmid-induced tumors (Bomhoff *et al.*, 1976), and can be specifically metabolized by agrobacteria growing in the incited tumors (Petit *et al.*, 1970; Petit and Tempé, 1978; Schell *et al.*, 1979; Tempé *et al.*, 1980). The Ti plasmids are currently classified into three groups according to the type of opine they induce in the incited tumors as octopine, nopaline or agropine Ti plasmids (Guyon *et al.*, 1980).

The T-DNA in octopine tumors consists of two distinguishable segments: TL-DNA and TR-DNA (Thomashow *et al.*, 1980; De Beuckeleer *et al.*, 1981). TL-DNA, which is essential and sufficient for octopine crown gall formation, codes for eight polyadenylated transcripts, each expressed from an individual promoter (Gelvin *et al.*, 1982; Willmitzer *et al.*, 1982). One of these transcripts (transcript 3) was shown to code directly for the enzyme octopine synthase (Schröder *et al.*, 1981). The nucleotide sequence of this gene was elucidated and both the 5′ and the 3′ ends of the transcript were precisely identified by S1 nuclease mapping (De Greve *et al.*, 1982). The 5′ end of the transcript coding for octopine synthase is located close to the right border of TL-DNA at a distance of 350 – 400 bp. This gene is transcribed from right to left (Willmitzer *et al.*, 1982).

The T-DNA of nopaline Ti plasmids codes for up to 13 polyadenylated transcripts (Bevan and Chilton, 1982; Willmitzer *et al.*, 1983). The region responsible for tumor maintenance is highly homologous between octopine TL-DNA and nopaline T-DNA (Engler *et al.*, 1981). Transcripts and gene functions determined by this conserved 'core' region are common in octopine and nopaline tumors (Joos *et al.*, 1983; Willmitzer *et al.*, 1983). Two different opines were detected in nopaline tumors: agrocinopine (Ellis and Murphy, 1981) and nopaline (Petit *et al.*, 1970). The nopaline synthase gene has been localized by genetic and transcript mapping on *Hind*III fragment 23 of plasmids pTiC58 and pTiT37 (Holsters *et al.*, 1980; Hernalsteens *et al.*, 1980; Joos *et al.*, 1983; Willmitzer *et al.*, 1983). DNA sequencing of *Hind*III fragment 23 localized the nopaline synthase gene (Depicker *et al.*, 1982) and the precise position of the right T-DNA borders within *Hind*III fragment 23 (Zambryski *et al.*, 1982).

To determine whether all signals essential for the expression of the opine synthase genes *in vivo* are located between the 5′ initiation site of the opine genes and the junction site with plant DNA or whether expression of these genes is activated by plant DNA sequences, we constructed octopine and nopaline synthase genes with different lengths of sequences upstream of the 5′ initiation site and reinserted them in the T-DNA of the Ti plasmids. This approach allowed us to delimit which sequences are important for the *in vivo* expression of the octopine and nopaline synthase genes, and to demonstrate that the plasmid-derived sequences contain all signals necessary for expression in plants.

Results

Expression of the octopine synthase gene in nopaline tumors

Construction of intermediate vectors pGV761, pGV762 and pGV763. The precise number of base pairs in the DNA region between the 5′ initiation site of the octopine synthase transcript (De Greve *et al.*, 1982) and the right T-region border sequence (Holsters *et al.*, 1983) has been determined and was found to be 402 (Figure 1a). Therefore, sequences essential for the expression of octopine synthase must either be located in this sequence, or activation of the promoter occurs by junction of the 5′ end of the *ocs* gene with plant

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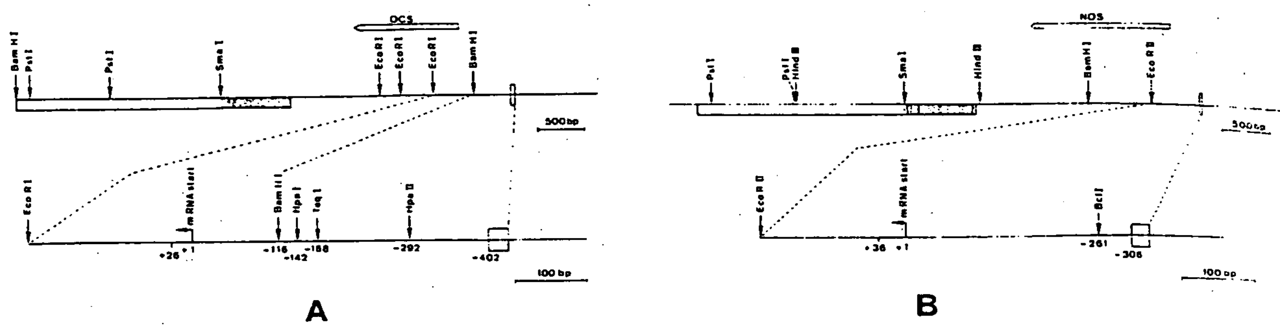


Fig. 1. (A) In the upper part the *Bam*HI fragment 17a and sequences up to the border (white box) are indicated, and the location and transcription polarity of the octopine synthase gene. The white bar shows the homology region between *Bam*HI fragment 17a and the nopaline T-DNA. The hatched portion of the white bar shows the homology region of 750 bp between plasmids pGV761, pGV762, pGV763 and the nopaline Ti plasmid. In the lower part the position of the restriction sites used in this study are indicated with regard to the transcription start of the octopine synthase gene. (B) In the upper part the *Hind*III fragments 23 and 31, and part of the *Hind*III fragment 22, are indicated (Depicker *et al.*, 1980). The position and transcription polarity of the nopaline synthase gene located in *Hind*III fragment 23 and the homology region with *Bam*HI fragment 17a of the octopine Ti plasmid pTiAch5 are shown. In the lower part the position of the *Bcl*I site is indicated with regard to the transcription start of the nopaline synthase gene.

DNA.

To test which of these possibilities is valid, intermediate vectors containing the octopine synthase gene and different lengths of 5′-flanking sequences (respectively −116 bp, −168 bp and −292 bp from the transcription start; Figure 1a) were constructed and introduced into the nopaline Ti plasmid C58. If the first possibility is correct, these constructions should allow us to delimit the sequences involved in the *in vivo* expression of the octopine synthase gene. The different steps in the construction of the intermediate vectors are outlined in Figure 2.

Isolation of co-integrated Ti plasmids. As the homology region between plasmids pGV761, pGV762 and pGV763 (Figure 1), and the nopaline Ti plasmid is only 750 bp, we envisaged, to avoid problems of recombination, using the homology of 1270 bp between the *amp* gene located on pBR322 and the transposon Tn*1*, inserted into the T-DNA of the nopaline Ti plasmid C58 (Joos *et al.*, 1983; Inzé *et al.*, in preparation).

For this purpose, we selected the plasmids pGV3300 and pGV3305. In pGV3300 a Tn*1* is inserted in *Hind*III fragment 23 just outside the nopaline synthase gene, while in pGV3305 the Tn*1* insertion is located in the nopaline synthase gene. The intermediate vectors pGV761, pGV762 and pGV763 were mobilized from *Escherichia coli* to *Agrobacterium* strains GV3101 (pGV3300) and GV3101 (pGV3305) with the helper plasmids R64*drd*11 and pGJ28 (Van Haute *et al.*, 1983). In all cases, Km^R transconjugants were isolated with a frequency of 10^{-6}–10^{-7}. Several co-integrate plasmids resulting from a single cross-over were analyzed by DNA/DNA hybridization to confirm their physical structure (data not shown). Recombination always occurred within the homology region common to pBR322 and Tn*1*.

Properties of the co-integrated plasmids. Sunflower hypocotyls and tobacco W38 plants were inoculated with the *Agrobacterium* strains containing these different co-integrates. The different primary tumor tissues were subsequently analyzed for octopine synthase activity (Otten and Schilperoort, 1978). No octopine synthase activity was detected in sunflower and tobacco tumors induced by the *Agrobacterium* strains containing the co-integrated plasmids pGV2290 (pGV3300::pGV761) and pGV2291 (pGV3305::pGV761). Furthermore, in tumors induced by *Agrobacterium* strains containing the co-integrated plasmids pGV2292 (pGV3300::pGV762) and pGV2293 (pGV3305::pGV762), again no detectable octopine synthase activity could be detected. On the contrary, in sunflower and tobacco tumors induced with *Agrobacterium* strains containing the co-integrated plasmids pGV2294 (pGV3300::pGV763) and pGV2295 (pGV3305::pGV763), octopine synthase activity was detected (Figure 3). The level of activity in these tumors was equal to that found in tumors induced by the *Agrobacterium* strain C58C1 containing an octopine Ti plasmid (pTiB6S3Tra^c).

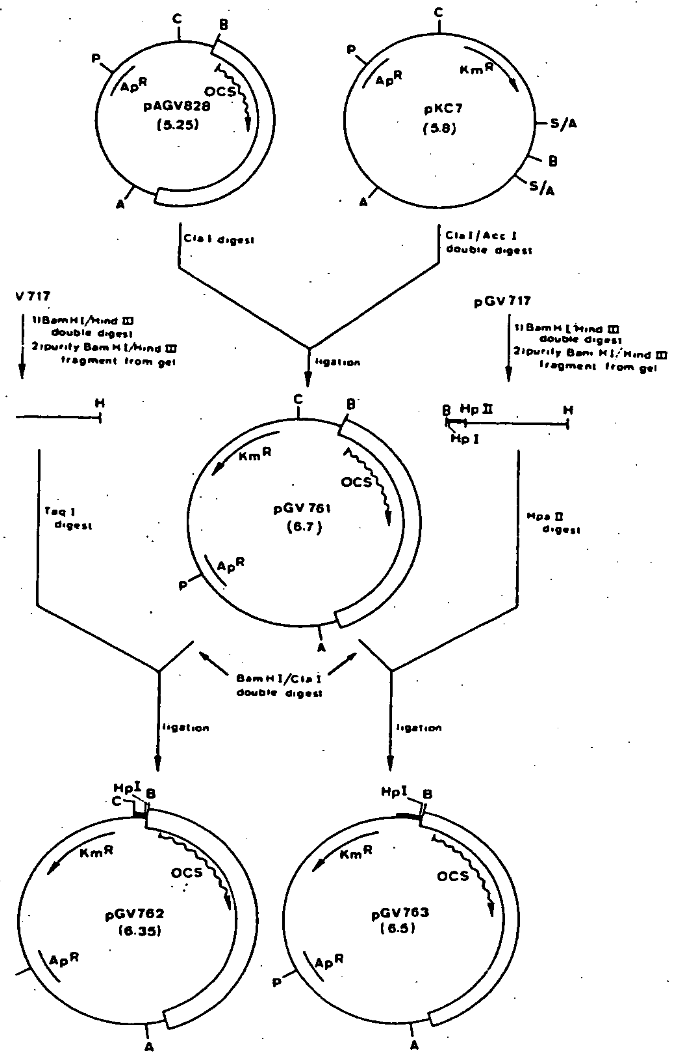
Expression of the nopaline synthase gene in octopine tumors

We have studied the expression of the nopaline synthase gene by a similar approach. DNA sequence analysis showed that the nopaline synthase gene is entirely encoded by the *Hind*III fragment 23 of pTiC58 (Depicker *et al.*, 1982). Furthermore, genomic blotting analysis of nopaline tumor tissues (Lemmers *et al.*, 1980) showed that this *Hind*III-23 fragment is a border fragment. Genomic clones isolated from different nopaline tumor tissues (Zambryski *et al.*, 1980, 1982; Holsters *et al.*, 1982) allowed us to determine the exact end points of the T-DNA in crown gall lines. The right T-DNA/plant DNA border is located only 305 bp (Figure 1b) from the start of the nopaline synthase transcript (Depicker *et al.*, 1982).

Construction and properties of pGV2253 and pGV2254

Construction of intermediate vectors pGV705 and pGV706. To demonstrate that the expression of the nopaline synthase gene is independent of the formation of a junction to plant DNA sequences, and that all sequences involved in the *in vivo* expression of the nopaline synthase gene are present between the start of the mRNA and the end of the T-DNA, we constructed an intermediate vector in which the sequences between the *Hind*III site and the *Bcl*I site (position −261; Figure 1b) of the *Hind*III fragment 23 have been deleted and replaced by the Sm^R gene of R702. This substitution deletes the 22-bp consensus sequence (position −305; Figure 1b) which is found at the ends of nopaline and octopine T-regions, and which might play a key role in the integration of the T-region into the plant genome (Zambryski *et al.*, 1980, 1982; Simpson *et al.*, 1982; Yadav *et al.*, 1982; Holsters *et al.*, 1982, 1983). The construction of the intermediate vector pGV705 is shown in Figure 4.

pGV705 consists of *Eco*RI fragment 12 of pTiAch5 in which the internal *Hind*III-36a fragment has been substituted



. Construction of intermediate vectors pGV762 and pGV763. The *ClaI* fragment of pKC7 containing the Km gene was ligated to *ClaI*-ed pAGV828. After ligation and selection on ApKm plates, recom-s were screened for the orientation of the Km-resistant fragment by digestion with *ClaI* and *BamHI*. A recombinant plasmid pGV761 gested with *BamHI* and *ClaI*, and ligated to the purified *HindIII*-II fragment of pGV717, which contains sequences 5′ upstream of the II site at −116 in the promoter region of the octopine synthase gene 1; Holsters *et al.*, 1983), digested with either *TaqI* or *HpaII*. By ing recombinant plasmids for the presence of a *HpaI* site (Figure 1), 52 and pGV763 were obtained. Abbreviations: A, *AccI*; B, *BamHI*; I; H, *HindIII*; HpI, *HpaI*; HpII, *HpaII*; P, *PstI*; S, *SalI*; T, *TaqI*.

e *HindIII-BclI* fragment of the nopaline *HindIII* frag-23 joined to the *BamHI-HindIII* fragment of plasmid 2 containing the Sm^R gene. This *HindIII* fragment in-l in the other orientation in the *EcoRI* fragment 12, is pGV706.

lation of pGV2253 and pGV2254. The intermediate vec-pGV705 and pGV706 were mobilized from *E. coli* to *bacterium* strain GV3000 carrying a transfer-constitutive S3 plasmid with the help of the plasmids R64*drd*11 and 3 (Van Haute *et al.*, 1983). Streptomycin-resistant *Agro-ium* strains were obtained in both cases with a joint er and recombination frequency of 10^{-6}. The Sm-nt transconjugants were tested directly for Km sensitivi-

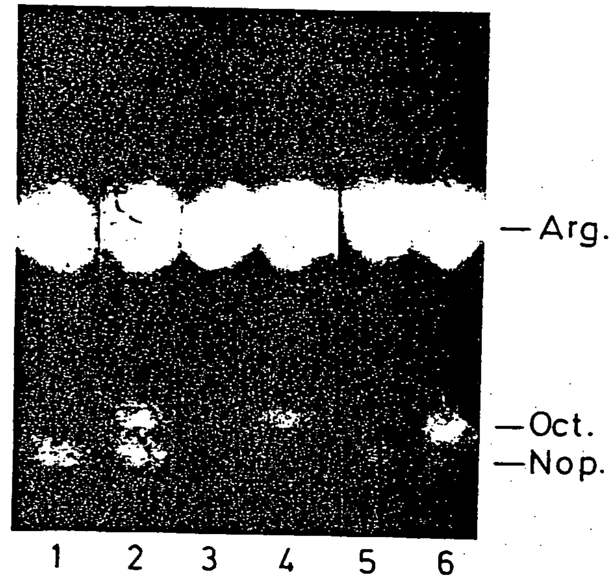


Fig. 3. Detection of octopine in tumors induced with *Agrobacterium* strains containing the mutant plasmids. 2 μl of extracts of tumor tissue before (**lanes 1, 3, 5**) and after (**lanes 2, 4, 6**) 1 h incubation were spotted onto Whatman 3MM paper and subjected to electrophoresis. **Lanes 1 and 2:** extracts obtained from tissue infected with *Agrobacterium* containing pGV2295; **lanes 3 and 4:** extracts obtained from tissue infected with *Agrobacterium* containing pGV2294; **lanes 5 and 6:** extracts obtained from tissue infected with *Agrobacterium* containing pGV2254.

ty. Three percent of the Sm^R transconjugants were Km-sensitive and were double recombinants. The structure of two plasmids pGV2253 and pGV2254 was confirmed by DNA-DNA hybridization (data not shown).

Properties of pGV2253 and pGV2254. Agrobacterium strain containing either pGV2253 or pGV2254 were used to incite tumors on tobacco plants. These tumors synthesize nopaline and octopine (Figure 3), but no mannopine or agropine could be detected. This observation indicates that the deletion substitution of the small *HindIII* fragment 36a abolishes the synthesis of mannopine and agropine.

Morover, since the sequences between the end of the nopaline T-DNA (position −305) and the *BclI* site (position −261) have been deleted and replaced by the Sm^R gene of pR702, the 5′-flanking region of the nopaline synthase gene in this construction is separated from TR sequences located to the right (in pGV2253) or to the left (in pGV2254), by the Sm^R insert fragment. Therefore, all the sequences involved in the *in vivo* expression of the nopaline gene must lie within the 5′-flanking region between the start of transcription and the *BclI* site (position −261).

Discussion

Most of the understanding of the regulatory events controlling gene expression in higher eukaryotes is derived from studies with animal viruses. Several eukaryotic promoters have been examined both by DNA sequencing and by *in vitro* and *in vivo* analysis of mutants. These studies have led to the identification of the so-called Goldberg-Hogness or TATA box, a signal that is involved in the precise positioning of 5′ RNA ends of genes transcribed by RNA polymerase II (Breathnach and Chambon, 1981; Shenk, 1981). Although the TATA box seems to be both necessary and sufficient for

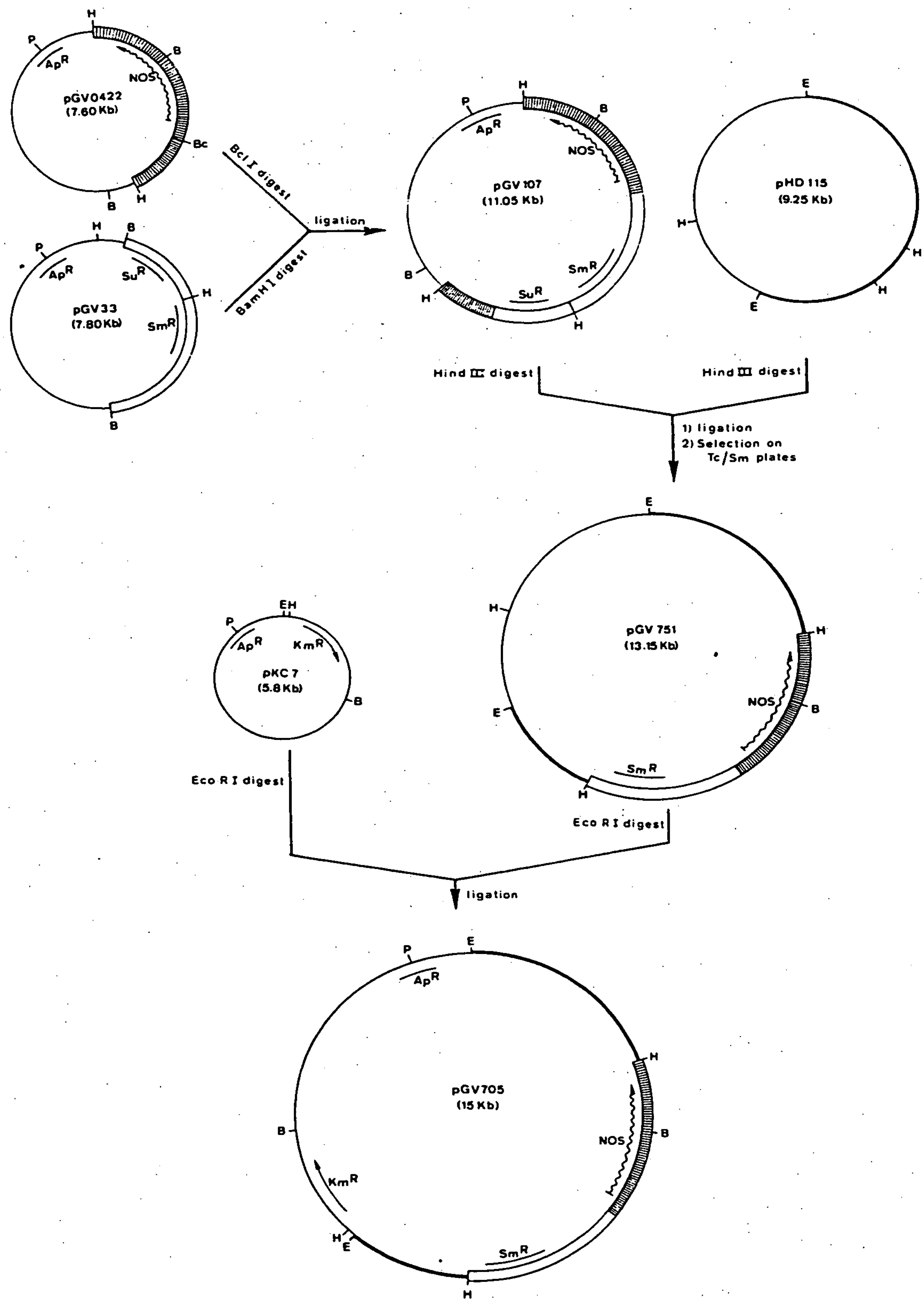


Fig. 4. Construction of the intermediate vector pGV705. Plasmids pGV0422 was linearized with *Bcl*I and ligated to *Bam*HI-digested pGV33. After transformation, recombinants were selected on Ap/Sm plates. One of the recombinants, pGV107, was digested with *Hin*dIII and ligated to *Hin*dIII-digested pHD115, containing the *Eco*RI fragment 12 of pTiAch5. After selection on Tc/Sm plates a recombinant, pGV751, was digested with *Eco*RI and ligated to *Eco*RI-digested pKC7, making it possible to use the mobilizing method described by Van Haute *et al.* (1983). Indeed, pGV751, a pACYC184 derivative, cannot be mobilized by pGJ28 and R64*drd*11.

Table 1. Bacterial strains and plasmids

	Antibiotic resistance	Characteristics	Dimension (kb)	Origin
Strains				
E. coli				
K514		*thr leu thi hsd*R		Colson *et al.* (1965)
A. tumefaciens				
GV3101		Rif^R derivative of C58, cured for pTiC58		Van Larebeke *et al.* (1974)
GV3105		Ery^R Cml^R derivative of C58, cured for pTiC58		Holsters *et al.* (1980)
Plasmids				
pKC7	Ap Km	*Hind*III-*Bam*HI of Tn*5* in pBR322	5.8	Rao and Rodgers (1979)
pGV0153	Ap	*Bam*HI-8 of pTiAch5 in pBR322	11.6	De Vos *et al.* (1981)
pGV0201	Ap	*Hind*III-1 of pTiAch5 in pBR322	16.9	De Vos *et al.* (1981)
pGV0422	Ap	*Hind*III-23 of pTiC58 in pBR322	7.6	Depicker *et al.* (1980)
pGV705	Ap Km Sm	*Hind*III fragment containing the *nos* gene and Sm/Sp marker of R702 in *Eco*RI-12	15	This work
pGV706	Ap Km Sm	*Hind*III fragment containing the *nos* gene and Sm/Sp marker of R702 in *Eco*RI-12, but in opposite direction	15	This work
pGV717	Ap	*Hind*III-*Bam*HI fragment of gcl rGV1-1 in pBR322	5.1	Holsters *et al.* (1983)
pAGV828	Ap	*Bam*HI-*Sma*I of pGV99 in pBR322	5.25	Herrera-Estrella *et al.* (1983)
pGV761	Ap Km	*Cla*I-*Acc*I of pKC7 in pAGV828	6.7	This work
pGV762	Ap Km	*Taq*I-*Bam*HI of pGV717 in pGV761	6.35	This work
pGV763	Ap Km	*Hpa*II-*Bam*HI of pGV717 in pGV761	6.5	This work
pGV33	Ap Sm/Sp Su	3.5 kb *Bam*HI fragment of R702 in pBR322	7.7	J. Leemans
pHD115	Tc	*Eco*RI-12 fragment of pTiAch5 in pACY184	9.25	J. Velten
R702	Km Sm/Sp Tc Su Hg	P-type plasmid	69.0	Hedges and Jacobs (1974)
R64drd11	Tc Sm	Iα-type plasmid, transfer-derepressed derivative of R64	109.0	Meynell and Datta (1967)
pGJ28	Km/Nm	Cda^+ Ida^+ ColD replicon carrying ColE1 *mob* and *bom*	9.7	Van Haute *et al.* (1983)
pGV3100	–	pTiC58, derepressed for autotransfer	212	Holsters *et al.* (1980)
pGV3300	Ap	pGV3100::Tn*1*	215	Joos *et al.* (1983)
pGV3305	Ap	pGV3100::Tn*1*	215	D. Inzé
pTiB6S3Tra[c]		pTiB6S3, derepressed for autotransfer	192	Petit *et al.* (1978)

ıccurate initiation of transcription *in vitro* (Corden *et al.*, 980; Wasylyk *et al.*, 1980), regions further upstream are reıuired for efficient *in vivo* transcription (Grosschedl and 3irnstiel, 1980; Benoist and Chambon, 1980; McKnight *et ıl.*, 1981; Grosveld *et al.*, 1982; Weiher *et al.*, 1983). Recenty, a detailed analysis of the promoter of the herpes simplex hymidine kinase (TK) gene (McKnight and Kingsbury, 1982) esulted in an identification of three essential regions within 05 bp upstream of the RNA initiation site.

In higher plants, on the contrary, little is known about seuence signals controlling gene expression. In octopine and opaline crown gall tumor tissues, the T-DNA is transcribed y RNA polymerase II (Willmitzer *et al.*, 1981a), and encodes set of well-defined polyadenylated transcripts. Therefore, ıe T-DNA genes can serve as models for defining transcripional and translational control sequences in nuclear, proteinɔding plant genes. In a first approach, we have attempted to etermine which are the minimal 5′ upstream sequences involved in the *in vivo* expression of these opine genes. Deletion of sequences upstream of position −170 of the octopine synthase gene greatly reduces or abolishes the gene expression, while deletion of sequences upstream of position −294 does not interfere with a wild-type level of gene expression. In this sequence of 125 bp an essential region controlling the expression of the octopine synthase gene might be located. Also in the case of the nopaline synthase gene, the 5′ sequences downstream of position −261 contain all the information necessary for the *in vivo* expression of this gene. Therefore, the estimated maximum size of the octopine and nopaline synthase gene promoters are 295 bp and 261 bp, respectively. Although the DNA sequences directly involved in the expression of the opine synthase genes in plant cells are not defined in this study, and identification of these sequences could help in the elucidation of the mechanisms of plant cellular gene control, the results described above clearly demonstrate that the expression of octopine and nopaline synthase genes is

determined directly by their 5′ upstream flanking sequences and is independent of the direct vicinity of the plant DNA sequences.

Materials and methods

Bacterial strains and plasmids

Bacterial strains and plasmids are listed in Table I.

Media and culture conditions

Luria broth (LB) and minimal A (minA) media were as described (Miller, 1972). Nitrogen-free medium for the use of octopine or nopaline as sole nitrogen source were as described (Bomhoff *et al.*, 1976). *E. coli* cultures were grown at 37°C and *A. tumefaciens* at 28°C. Antibiotic concentrations used for *E. coli* and *A. tumefaciens* were respectively, carbenicillin (Cb), 100 μg/ml; streptomycin (Sm), 20 μg/ml and 300 μg/ml; spectinomycin (Sp), 50 μg/ml and 100 μg/ml; kanamycin (Km), 25 μg/ml; rifampicin (Rif), 100 μg/ml; erythromycin (Ery), 50 μg/ml for *Agrobacterium*; chloramphenicol (Cml), 25 μg/ml for *Agrobacterium*.

Plasmid isolation

Plasmids were prepared from *E. coli* by density gradient centrifugation in a CsCl-ethidium bromide gradient of cleared SDS lysates (Betlach *et al.*, 1976). For screening of recombinant plasmids, plasmid DNA was obtained from 10 ml cultures as described (Klein *et al.*, 1980).

DNA analysis

Restriction enzyme analysis, agarose gel electrophoresis, conditions for DNA ligation and transformation of competent *E. coli* were as described (Depicker *et al.*, 1980). DNA fragments were extracted from low-gelling agarose gels as described (Wieslander, 1979). Total DNA of Ti plasmid-containing *Agrobacterium* strains was prepared, digested, separated on agarose gel, transferred to nitrocellulose paper, and hybridized against radioactively labeled recombinant plasmids as described (Dhaese *et al.*, 1979).

Induction and culture of crown gall tumors

Sterile 1-month-old tobacco plants (Wisconsin 38 or SR1) were decapitated and infected with freshly grown agrobacteria. Three weeks later, tumors were excised and transferred to hormone-free Murashige and Skoog medium (Murashige and Skoog, 1962) containing sucrose (30 g/l) and 0.5 mg/ml HR756 (Hoechst A.G.). The tumor tissues, transferred every month, were usually free of bacteria after three transfers, and were further cultivated on antibiotic-free Murashige and Skoog medium. Sunflower hypocotyl segments were inoculated as described by Petit and Tempé (1978).

Detection of opines in plant tumor tissue

Octopine and nopaline detection. The presence of octopine or nopaline in tumor tissue was tested as described by Leemans *et al.* (1981). Octopine or nopaline synthase activity were determined *in vitro* according to Otten and Schilperoort (1978).

Agropine and mannopine detection. Agropine and mannopine were detected in tumor tissue as described by Leemans *et al.* (1981).

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